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Global Distribution of Panton-Valentine Leukocidin-positive Methicillin-resistant *Staphylococcus aureus*, 2006

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We determined the *agr* type, multilocus sequence type, protein A gene type (*spa* typing), toxin gene profile, and antimicrobial drug resistance profile of 469 isolates of Panton-Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus* isolates (PVL-positive CA-MRSA). The isolates had been collected from around the world from 1999 through 2005 by the French National Reference Center for Staphylococci. We found that some continent-specific clones described in 2003, such as clone ST8, have now spread all over the world. Likewise, some PVL-positive CA-MRSA have spread to several countries on various continents. New clones have emerged (e.g., ST377) on new genetic backgrounds. PVL-positive CA-MRSA that were usually susceptible to most antistaphylococcal antimicrobial agents have acquired new resistance determinants (e.g., to gentamicin) in certain countries. The major trait shared by all these clones is a short staphylococcal chromosomal cassette *mec* element of type IV or V.

By definition, community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains infect patients with no risk factors for acquiring an MRSA strain of hospital origin. CA-MRSA infections usually affect previously healthy young patients (1). These infections are mostly of skin and soft tissue, but deep-seated infections such as necrotizing pneumonia and disseminated invasive osteomyelitis have been described (2). Many CA-MRSA isolates produce Panton-Valentine leukocidin (PVL) and harbor a type IV staphylococcal chromosomal cassette *mec* (SCC*mec*) element, but some isolates harbor-

ing the SCC*mec* V element have been reported (3). PVL-positive CA-MRSA clones have spread throughout the world (4).

In 2003, Vandenesch et al. described continent-specific PVL-positive CA-MRSA clones (mainly on an *agr3* background) and characterized them by their sequence type (ST) (4). The main European clone, ST80, was detected in France, Switzerland, the Netherlands, England, Belgium, and Germany (5,6), but also in northern Europe (e.g., Denmark), where MRSA strains are rare in hospitals (7). One of the most prevalent PVL-positive CA-MRSA clones in the United States (USA300) belongs to ST8 (8); other US clones include USA400 (ST1), USA1000 (ST59), and USA1100 (ST30) (9). ST30 is also a major clone in Asia and Oceania (10,11) and is referred to as the South West Pacific clone (11). In Singapore, an international travel hub, several clones belonging to ST80, ST30, and ST59 have been reported (12). The prevalence of PVL-positive CA-MRSA varies considerably from continent to continent. In the United States, MRSA were isolated from 50% of patients with skin and soft-tissue infections seen in emergency departments of 11 cities (97% of isolates belonged to clone USA300) (13). In Europe, the prevalence is lower, at ≈1–3% (14).

Since 1999, the French National Center for Staphylococci has characterized 469 PVL-positive CA-MRSA isolates collected throughout the world. The isolates were typed by multilocus sequence type (MLST), protein A gene type (*spa* typing), antimicrobial drug resistance profiling, and toxin and resistance gene analysis. We describe the evolution and spread of PVL-positive CA-MRSA clones since their initial description.

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Materials and Methods

Bacterial Isolates

From 1999 through 2005, the French National Reference Center for Staphylococci received 469 PVL-positive CA-MRSA isolates from 17 countries. These isolates were voluntarily sent to the center for PVL for gene detection and genomic characterization (clone designation). A single strain was selected per patient. Twenty-two isolates corresponded to an outbreak linked to a maternity unit that occurred in France in 2002–2003.

DNA Extraction and Identification of *agr* Alleles

The strains were grown on brain–heart infusion agar or in brain–heart infusion broth at 37°C overnight. Genomic DNA was extracted with a standard procedure (15). Amplification of *gyrA* was used to confirm the quality of each DNA extract and the absence of PCR inhibitors. All PCR products were analyzed by electrophoresis on ethidium bromide–stained 1% agarose gels (Sigma, Lyon, France). The *agr* group (*agr* 1–4) was determined by PCR as previously described (15).

Detection of the *mecA* Gene and SCCmec Typing

The *mecA* gene (coding for methicillin resistance) was detected by PCR as previously described (3). The staphylococcal chromosomal cassette *mec* (SCCmec I–IV) was detected as described by Oliveira et al. (16), and SCCmec type V was detected as previously described (3). The following reference strains were used as controls: COL (SCCmec I), BK2464 (SCCmec II), HU106 (SCCmec III), and BK2529 (SCCmec IV).

Detection of Toxin Genes

Using PCR, we determined the presence of 22 specific staphylococcal virulence genes, as described previously (15,17). We detected sequences specific for staphylococcal enterotoxin genes (*sea-e*, *seh*, *sek*, *sem*, *seo*), as well as the toxic-shock syndrome toxin gene (*tst*), exfoliative toxin genes (*eta*, *etb*, *etd*), PVL genes (*lukS*–PV–*lukF*–PV), LukE–lukD leukocidin genes (*lukE*–*lukD*), the class F lukM leukocidin gene (*lukM*), hemolysin genes (gamma [*hlg*], gamma variant [*hlgv*] and β [*hlb*]), and epidermal cell differentiation inhibitor genes (*edinA/B/C*).

Antimicrobial Susceptibility Testing

MICs of penicillin, oxacillin, cefoxitin, kanamycin, tobramycin, gentamicin, erythromycin, clindamycin, tetracycline, pristinamycin, ofloxacin, fusidic acid, vancomycin, teicoplanin, fosfomycin, trimethoprim/sulfamethoxazole, rifampin, mupirocin, quinupristin/dalfopristin, and linezolid were determined by using the standard agar dilution technique, as recommended by the

French Society for Microbiology. Structural genes for resistance to tetracycline, aminoglycosides, and macrolide-lincosamide-streptogramin (18) were identified by PCR. DNA was amplified in a model 2400 thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) with *Taq* (Qbiogene, Inc., Carlsbad, CA, USA) or *Pfu* (Stratagene, La Jolla, CA, USA) DNA polymerase, as recommended by the manufacturers. PCR elongation times and temperatures were adjusted to the expected size of the PCR product and to the nucleotide sequences of the primers, respectively.

spa Typing

spa typing was performed on PVL-positive MRSA isolates as previously described (19). The *x* region of the *spa* gene was amplified by PCR. *spa* types were determined with the Ridom Staph Type software (Ridom GmbH, Würzburg, Germany), which automatically detects *spa* repeats and assigns a *spa* type according to Harmsen et al. (20) (<http://spaserver.ridom.de>). When the recently developed algorithm BURP (Based Upon Repeat Patterns) was applied, *spa* types were clustered into different groups with calculated cost between members of a group ≤ 6 . *spa* types shorter than 3 repeats were excluded from analysis because no reliable deduction about ancestries can be made from these types. In addition to point mutation events, the new algorithm takes all other modifications that can occur (repeat or duplication or deletion) into account when the relatedness of different *spa* types is calculated. Because of speed constraints, a heuristic version of the EDSI-Alignment (Excisions, Duplications, Substitutions, Insertions), as described by Sammeth et al., was used (21). BURP *spa* clonal complexes (*spa*-CC) were automatically assigned by the Ridom Staph Type software (www.ridom.de/staphtype/).

MLST

MLST was performed on representative strains of each clonal group, as described elsewhere (22). The allelic profile of each strain was obtained by sequencing internal fragments of 7 housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yqiL*) and entering them on the MLST home page (<http://saureus.mlst.net>), where 7 numbers depicting the allelic profile were assigned that defined a ST (22). Similar STs were grouped into clonal complexes (CC).

Results

agr and STs

The 469 PVL-positive CA-MRSA isolates were *agr* type 1 ($n = 46$, 9.8%), *agr*2 ($n = 9$, 1.9%), or *agr*3 ($n = 414$, 88.3%); none was *agr*4 (Table 1). The 469 PVL-positive isolates belonged to 11 STs: the *agr*1 isolates were ST8, ST59, ST22, ST766, or ST377; the *agr*2 isolates were all

ST5; and the *agr3* isolates were ST80, ST30, ST37, ST93, or ST1 (Table 1). None of the STs were shared by different *agr* types. The most frequent ST was ST80 ($n = 357$, 76.1%), which corresponded to the European clone.

***spa* Types and *spa* Clonal Complexes**

The *spa* types were specific for *agr* type and ST. Minor variations of *spa* types (deletions or duplications of SSR units) were observed in several isolates within the same ST. For instance, 9 *spa* types were recognized among the 357 ST80 isolates, but t044 was the major *spa* type ($n = 333$, 93.3%); 8 of these *spa* types belonged to the same *spa* CC. A unique *spa* CC corresponded to each ST, except for ST1 isolates, which formed 3 different *spa* CC (Table 1).

Geographic Origin and Spread

A previous study (4) showed a limited number of clones and a limited geographic distribution. Schematically, ST80 was detected in Europe, ST8 and ST1 in the United States, and ST30 in Oceania. The results of our study suggest intercontinental exchanges of several clones (Table 1): 1) the ST8 clone (USA300) from the United States toward Europe; 2) the ST1 clone (USA400) from the United States toward Europe and Asia; 3) the ST59 clone (USA1000) from the United States toward Asia; 4) the ST80 clone from Europe toward Asia (12); and 5) the ST30 clone from Oceania toward Europe. Countries with numerous international exchanges (e.g., Singapore) have the highest clonal diversity.

New clones have been detected since 2003. One, ST22, has been found in Europe only. Another new clone, ST766, which belongs to the same CC (CC22) as ST22, was found in Singapore (12). Clone ST377 (with a type V SCCmec) was reported simultaneously in Europe and Australia (3). Clone ST5 was detected in Europe only. Clone ST93 (the Queensland clone), described in Australia before 2003, has not yet been detected in other countries (11).

Toxin Gene Content

The toxin gene distributions were compared to determine the genetic background of the different clones with minor variations. For instance, ST80 isolates were all positive for *etd*, *lukS*-PV, *lukF*-PV, and *ednA/B/C*; very few lacked *lukDE* or *hlgv* or harbored *h1B* (Table 2). Superantigenic toxin genes were detected in isolates that belonged to the different STs, except for ST377, ST80, and ST93 (Table 2).

Antimicrobial Drug Resistance

Isolates of each ST were grouped according to the number of antimicrobial drug resistance determinants they

harbored. Initial PVL-positive CA-MRSA isolates were susceptible to most antimicrobial agents. For instance, 8 of the 25 ST8 isolates included in this study between 2003 through 2005 were resistant to penicillin and oxacillin alone, as were 17 of the 32 ST1 isolates and 18 of the 20 ST30 isolates (Online Appendix Table, available at www.cdc.gov/EID/content/13/4/594-appT.htm). ST80 isolates were initially resistant to penicillin, oxacillin, kanamycin, and tetracycline, and intermediate to fusidic acid. Since 2003, new antimicrobial resistance determinants have been acquired (e.g., gentamicin and ofloxacin). One ST8 isolate was resistant to penicillin, oxacillin, kanamycin, erythromycin, tetracycline, and ofloxacin; 1 ST1 isolate was resistant to penicillin, oxacillin, kanamycin, tobramycin, and gentamicin. A few ST80 isolates from Algeria were resistant to multiple antimicrobial agents. Most PVL-positive CA-MRSA strains with multiple antimicrobial resistant determinants were detected in Asia (Singapore, People's Republic of China) or Africa (Algeria).

Antimicrobial Resistance Genes

Antimicrobial resistance genes were sought in a subset of 153 ST80 isolates. The *aph3'-III* gene, which encodes high-level resistance to kanamycin and neomycin but also to amikacin and isepamycin, was detected in all 153 isolates (100%). The *tetK* efflux gene was detected in 125 (82%) of tetracycline-resistant isolates. The *ermC* gene, an erythromycin ribosome methylase, was detected in 61 (40%) of erythromycin-resistant isolates. The *far-1* gene was detected in 133 (87%) of fusidic acid-intermediate isolates.

SCCmec Types

SCCmec type was determined for 22 *agr1* isolates (10 ST8, 1 ST59, 1 ST22, and 10 ST377); 5 *agr2* isolates (ST5); 190 *agr3* isolates (179 ST80, 9 ST30, 2 ST93, and 7 ST1). All the isolates were SCCmec type IV, except for the 10 ST377 isolates, which were SCCmec type V.

Discussion

This study has several findings. First, the continent-specific clones of PVL-positive CA-MRSA described in 2003 by Vandenesch et al. (4) have now spread to other continents. For instance, the ST1 clone USA400 is now detected in Europe and Asia. Some PVL-positive clones, such as ST1 and ST30, can now be considered pandemic, as they are detected in America, Europe, and Asia. Second, on a given continent, PVL-positive CA-MRSA have spread from country to country. For instance, in Europe, PVL-positive CA-MRSA were recently detected in Slovenia, Romania, and Croatia. Third, new PVL-positive CA-MRSA clones are emerging in strains with different

Table 1. Geographic distribution of PVL-positive CA-MRSA clones according to their *agr*-type, ST, and *spa* type*

<i>agr</i> type	ST (CC)	No. (%)	<i>spa</i> type (<i>spa</i> CC)		Location		
					Detected before 2003	Newly detected (this work)	Other reports in literature
<i>agr1</i>		46 (9.8)					
	ST8 (8)	25 (54.3)			USA (4)	Netherlands, France, Spain, Switzerland, French Polynesia	N. Norway (23) Greece (24)
			t008 (s)	25 (100.0)			
	ST59	7 (15.2)			USA (4)	Netherlands, France, Singapore	Taiwan (25)
			t437 (8)	6 (85.7)			
			t216 (8)	1 (14.3)			
	ST22 (22)	3 (6.5)				Netherlands, Germany	
			t005 (4)	2 (66.7)			
			t310 (4)	1 (33.3)			
	ST766 (22)	1 (2.2)				Singapore	
	ST377	10 (21.7)				Netherlands, France, Greece, Switzerland, Australia	
			t355 (6)	9 (90.0)			
			t595 (6)	1 (10.0)			
<i>agr2</i>		9 (1.9)					
	ST5 (5)	9 (100.0)				France, Switzerland, Algeria	
			t311 (5)	5 (55.5)			
			t1277	3 (33.3)			
<i>agr3</i>			t450	1 (11.1)			
	ST80	414 (88.3) 357 (83.2)			France, Switzerland (4)	Algeria, Singapore, Romania, Germany, Belgium, Greece, Slovenia, Netherlands	Denmark (7), Sweden (7), northern Norway (23), England (26), Finland (27), Libya (28), Croatia (29), Scotland (7), Greece (24)
			t044 (1)	333 (93.3)			
			t131 (1)	9 (2.5)			
			t376 (1)	8 (2.2)			
			t639 (1)	2 (0.6)			
			t237 (1)	1 (0.3)			
			t1199 (1)	1 (0.3)			
			t1201 (1)	1 (0.3)			
			t1206 (1)	1 (0.3)			
			t1200 (†)	1 (0.3)			
	ST30 (30)	20 (4.8)			New Zealand, Western Samoa (4)	Netherlands, Australia, Japan, Switzerland, Singapore, China, French Polynesia	Sweden (30), Brazil (31), Uruguay (32), England (26), Hong Kong (10)
			t019 (2)	17 (75.0)			
			t021 (2)	1 (5.0)			
			t318 (2)	1 (5.0)			
			t1273 (2)	1 (5.0)			
	ST37 (30)	1 (0.2)				Netherlands	
			t914 (2)	1 (100.0)			
	ST93	4 (1.0)			Australia (4)		
			t202 (s)	4 (100.0)			
	ST1	32 (7.7)			USA (4)	France, Singapore	Switzerland (28), Canada (33)
			t128 (3)	18 (56.2)			
			t125 (3)	3 (9.4)			
			t558 (3)	1 (3.1)			
			t175 (7)	8 (25.0)			
			t1274 (7)	1 (3.1)			
			t1272 (s)	1 (3.1)			

*PVL, Panton-Valentine leukodin; CA-MRSA, community-acquired methicillin-resistant *Staphylococcus aureus*; agr, accessory gene regulator; ST, sequence type; CC, clonal complexes; spa CC, spa clonal complexes.

†Excluded because <3 repeats; s, singleton.

Table 2. Toxin gene content of PVL-positive community-acquired methicillin-resistant *Staphylococcus aureus* clones*

<i>agr</i> type	ST	No. (%)	Toxin genes always detected (100%)	Toxin genes sometimes detected (%)
<i>agr1</i>	ST8	46 (9.8)		
	ST59	25 (54.3)	<i>lukPV</i> , <i>lukDE</i>	<i>hlgv</i> (95.8), <i>sek</i> (91.7), <i>sed</i> (16.7), <i>seb</i> (4.2), <i>hIB</i> (4.2)
	ST22	7 (15.2)	<i>lukPV</i> , <i>hlgv</i>	<i>hIB</i> (87.5), <i>sek</i> (87.5), <i>seb</i> (62.5), <i>lukDE</i> (12.5)
	ST766	3 (6.5)	<i>sem</i> , <i>seo</i> , <i>lukPV</i> , <i>hlg</i>	
	ST766	1 (2.2)	<i>sem</i> , <i>seo</i> , <i>lukPV</i> , <i>hlg</i>	
<i>agr2</i>	ST 377	10 (21.7)	<i>lukPV</i> , <i>edinA/B/C</i> , <i>hIB</i> , <i>hlg</i>	
		9 (1.9)		
<i>agr3</i>	ST5	9 (100)	<i>sem</i> , <i>seo</i> , <i>lukPV</i> , <i>lukED</i> , <i>hlgv</i>	<i>edinA/B/C</i> (55.5)
		414 (88.3)		
	ST80	357 (83.2)	<i>etd</i> , <i>lukPV</i> , <i>edinA/B/C</i>	<i>lukDE</i> (99.7), <i>hlgv</i> (99.7), <i>hIB</i> (0.8)
	ST30	20 (4.8)	<i>sem</i> , <i>seo</i> , <i>lukPV</i> , <i>hlg</i>	<i>sek</i> (5.0), <i>tst</i> (5.0)
	ST37	1 (0.2)	<i>sec</i> , <i>sem</i> , <i>seo</i> , <i>tst</i> , <i>lukPV</i> , <i>hlg</i>	
	ST93	4 (1)	<i>lukPV</i>	
	ST1	32 (7.7)	<i>lukPV</i> , <i>seh</i> , <i>lukDE</i> , <i>hlgv</i>	<i>sea</i> (78.1), <i>sec</i> (68.7), <i>sek</i> (68.7), <i>seb</i> (25.0), <i>edinA/B/C</i> (3.1)

*PVL, Panton-Valentine leukocidin; ST, sequence type; *lukPV*, PVL genes; *lukDE*, LukE–LukD leukocidin genes; gamma (*hlg*), gamma variant (*hlgv*), and β (*hIB*) hemolysin genes; *sea* to *see*, *seh*, *sek*, *sem*, *seo*, staphylococcal enterotoxin type A to E, H, K, M, and O genes, respectively; *tst*, toxic shock toxin gene; *eta*, *etb*, *etd*: exfoliative toxin type A, B, and D genes, respectively; *edinA/B/C*, epidermal cell differentiation inhibitor; *agr*, accessory gene regulator.

genetic backgrounds. While most of the clones described in 2003 by Vandenesch et al. (4) had an *agr3* background, the newly described clones are *agr1* or *agr2*. Fourth, PVL-positive CA-MRSA, which were initially susceptible to most antistaphylococcal antimicrobial agents, have acquired new antimicrobial resistance determinants, to gentamicin and ofloxacin, for instance.

The global ST distribution of PVL-positive CA-MRSA isolates in this study depends, of course, on the sources of the isolates received by the French National Reference Center for Staphylococci and does not reflect the current epidemiology. Our collection represents a passive surveillance study and is related to the increased attention paid to CA-MRSA by certain regions. Nevertheless, our results agree with other reports which confirm that ST80 is mainly found in Europe (e.g., Denmark [7], Finland [27], and Greece [24]), but also in Libya (28); ST30 is pandemic (34).

PVL-negative, hospital-acquired MRSA belong to 5 major CCs (CC5, CC8, CC22, CC30, CC45). PVL-positive CA-MRSA of the same clonal classes were also detected in our study, with the exception of CC45, but the PVL + MRSA strains showed a broader CC diversity. For instance, none of the ST80 isolates belonged to CCs harboring hospital strains. PVL-positive CA-MRSA are gradually causing an increasing number of hospital-acquired infections in countries, such as the United States, where their prevalence is high (35). Kourbatova et al. reported that, during the period 2003–2004, five prosthetic joint infections were caused by USA300 strains (36).

The worldwide spread of PVL-positive CA-MRSA is likely related to international travel. ST80 isolates recovered in France were mainly detected in patients who were originally from Algeria, a country that reported a high rate of community- and hospital-acquired infections due to

ST80 isolates in 2006 (37). Maier et al. recovered ST22 strains from Turkish migrants in Germany (38). Acquisition of new antimicrobial resistance determinants could be related to misuse of antimicrobial agents; the spread of multidrug-resistant strains could be facilitated by poor hygiene, regardless of country.

It is not known whether PVL-positive CA-MRSA clones arose through acquisition of the PVL phage by strains with a methicillin resistance background or, conversely, through acquisition of the SCCmec element by strains with a PVL-positive background. On analyzing the database of the French National Reference Center for Staphylococci, which contains >5,000 toxin gene profiles, we found isolates that were related to the PVL-positive MRSA clone ST80 but lacked either the PVL genes (5 isolates) or the *mecA* gene (7 isolates) (data not shown). These isolates, like the ST80 clone, were *agr3*, *etd*+, *edinA/B/C*+, 1 isolate (PVL– *mecA*+) was ST80, and another (PVL+ *mecA*–) was ST635 (a single-locus variant of ST80). These “atypical” isolates were discovered in Algeria, Switzerland, and France. We are unable to state whether they are ancestors or descendants of the most prevalent strains.

Deep-seated infections due to PVL-positive *S. aureus* can be extremely severe. For example, necrotizing pneumonia carries a mortality rate close to 75% (39). Whether the pathogenesis of these acute infections is related to the effect of PVL alone or in combination with other virulence factors such as superantigenic toxins is unclear. We found that some PVL-positive CA-MRSA clones (ST80) lacked any superantigenic toxin genes. Among the *S. aureus* virulence factors (not screened for here), ST30 strains harbor the *bbp* gene, which encodes bone sialoprotein (34). The SCCmec elements detected in our collection were type IV or V and corresponded to the smallest SCCmec element.

PVL-positive CA-MRSA are usually susceptible to most antistaphylococcal antimicrobial agents. Clone ST80 is usually resistant to tetracycline (mediated by the *tetK* gene), intermediate to fusidic acid (*farI* gene), and resistant to kanamycin (*aph3'-III* gene). We observed the emergence of rare isolates with multiple resistances to antimicrobial agents such as gentamicin and ofloxacin. From the therapeutic viewpoint, all the isolates were susceptible to trimethoprim-sulfamethoxazole, glycopeptides, and linezolid.

The involvement of PVL in CA-MRSA infections has not been proved in mouse sepsis and abscess models developed by Voyich et al.: isogenic PVL-negative strains of USA300 and 400 were as lethal as wild-type strains, and they caused comparable skin diseases (40). Other experiments have to be conducted to determine if PVL is secreted in such a model.

In summary, since 2003 we have observed an impressive worldwide spread of PVL-positive CA-MRSA clones initially described at the beginning of this decade, and we have also detected PVL-positive CA-MRSA strains of other lineages. To counter this emerging global threat to public health, systematic surveillance of both hospital and community isolates is required, together with measures designed to limit their spread.

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